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Moving into advanced nanomaterials. Toxicity of rutile TiO_2 nanoparticles immobilized in nanokaolin nanocomposites on HepG2 cell line

Maria João Bessa [a], Carla Costa [a,b,*], Julian Reinosa [c], Cristiana Pereira [a,b], Sónia Fraga [a,b], José Fernández [c], Miguel A. Bañares [d], João Paulo Teixeira [a,b]

[a] Department of Environmental Health, Portuguese National Institute of Health, Rua Alexandre Herculano, 321, 4000-055 Porto, Portugal
[b] EPIUnit - Institute of Public Health, University of Porto, Rua das Taipas 135, 4050-600, Porto, Portugal
[c] Electroceramic Department, Instituto de Cerámica y Vidrio, CSIC, Campus de Cantoblanco, Calle de Kelson, 5, 28049 Madrid, Spain
[d] Catalytic Spectroscopy Laboratory, Instituto de Catálisis y Petroleoquímica, ICP-CSIC, Madrid, Spain

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ABSTRACT

Immobilization of nanoparticles on inorganic supports has been recently developed, resulting in the creation of nanocomposites. Concerning titanium dioxide nanoparticles (TiO_2 NPs[1]), these have already been developed in conjugation with clays, but so far there are no available toxicological studies on these nanocomposites. The present work intended to evaluate the hepatic toxicity of nanocomposites (C-TiO_2[2]), constituted by rutile TiO_2 NPs immobilized in nanokaolin (NK[3]) clay, and its individual components.

These nanomaterials were analysed by means of FE-SEM[4] and DLS[5] analysis for physicochemical characterization. HepG2 cells were exposed to rutile TiO_2 NPs, NK clay and C-TiO_2 nanocomposite, in the presence and absence of serum for different exposure periods. Possible interferences with the methodological procedures were determined for MTT,[6] neutral red uptake, alamar blue (AB), LDH,[7] and comet assays, for all studied nanomaterials. Results showed that MTT, AB and alkaline comet assay were suitable for toxicity analysis of the present materials after slight modifications to the protocol. Significant decreases in cell viability were observed after exposure to all studied nanomaterials. Furthermore, an increase in HepG2 DNA damage was observed after shorter periods of exposure in the absence of serum proteins and longer periods of exposure in their presence. Although the immobilization of nanoparticles in micron-sized supports could, in theory, decrease the toxicity of single nanoparticles, the selection of a suitable support is essential. The present results suggest that NK clay is not the appropriate substrate to decrease TiO_2 NPs toxicity. Therefore, for future studies, it is critical to select a more appropriate substrate for the immobilization of TiO_2 NPs.

1. Introduction

Nanotechnology is growing at an increasingly rapid pace in the past years, revealing innovative and attractive nanomaterials with specific properties for several applications (Savolainen et al., 2013). Within the existing nanomaterials, the most used are the metal oxide nanoparticles (Djurisic et al., 2014), being titanium dioxide nanoparticles (TiO_2 NPs) the most extensively studied (both in concern to material characterization and toxic properties) due to their economic interest and unique properties, including large surface area, high stability, anticorrosive, ultra-violet (UV) absorption, whitening and opacifying characteristics and photocatalytic activity (Menard et al., 2011). Titanium dioxide is obtained from the iron mineral ilmenite, a naturally occurring crystal that can exist in three major crystalline forms (rutile, anatase and brookite) (Iavicoli et al., 2011; Park et al., 2014), but only rutile and

* Corresponding author at: Department of Environmental Health, Portuguese National Institute of Health, Rua Alexandre Herculano, 321, 4000-055, Porto, Portugal.
E-mail addresses: mjbessa8@gmail.com (M.J. Bessa), cstcosta@gmail.com (C. Costa), jjreinosa@icv.csic.es (J. Reinosa), cristianacostapereira@gmail.com (C. Pereira), teixeirafraga@hotmail.com (S. Fraga), jfernandez@icv.csic.es (J. Fernández), miguel.banares@csic.es (M.A. Bañares), jpft12@gmail.com (J.P. Teixeira).

[1] TiO_2 NPs: Titanium dioxide nanoparticles
[4] FE-SEM: Field Emission Scanning Electron Microscopy
[2] C-TiO_2: Rutile TiO_2 NPs immobilized in nanokaolin clay substrates
[5] DLS: Dynamic Light Scattering
[3] NK: Nanokaolin clay
[6] MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay
[7] LDH: Lactate dehydrogenase assay

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anatase are generally manufactured in titanium dioxide commercial applications (Iavicoli et al., 2011; Becker et al., 2014). Textile industries, biotechnology, pharmaceutical and cosmetics are prominent areas of TiO_2 NPs applications, leading to the increasing worldwide distribution of these nanoparticles (Gupta and Tripathi, 2011; Botelho et al., 2014). Due to the unavoidable presence of TiO_2 NPs, their toxicity have been widely studied; however, in the literature, the results have been contradictory (Iavicoli et al., 2012; Shi et al., 2013). For some researchers, TiO_2 NPs are considered to be a nontoxic mineral, being even used as negative control in many in vitro and in vivo studies (Liu et al., 2013). Despite that, others investigators demonstrate opposite effects. One main problematic associated with engineered nanoparticles such as TiO_2 NPs is that these materials can across the cell membrane, especially into the mitochondria, causing organelle dysfunction (Teodoro et al., 2011). Several authors suggests that TiO_2 NPs may induce higher toxicity potential, including the impairment of mitochondrial function, decrease of cell viability and proliferation, and an increase of oxidative stress leading to genotoxic effects and apoptotic events (Freyre-Fonseca et al., 2011; Iavicoli et al., 2011; Jaeger et al., 2012).

Owing to the distinctive properties derived by the nano-sized particles, some reports demonstrate that nanoparticles are more toxic when compared to larger micron-sized particles (Kocbek et al., 2010; Roy et al., 2014). This enhances the importance of an innovative area of nanotechnology: nanoarchitectonics. Nanoarchitectonics is a technology system which enables assembling nanostructure units into novel structure functionality through mutual interactions among units (Aono et al., 2012). This field of nanomaterials introduced innovations in the design and constructions systems in nanoelectronics, nanomachinery, energy conversion, and more recently in nanomedicine (Gonzalez-Alfaro et al., 2011; Kujawa and Winnik, 2013).

Recent advances lead to the development of new nanocomposites, namely nanoparticles immobilized in inorganic or organic substrates that, by presenting new physico-chemical features, must be tested with regards to their toxicological potential (Aono et al., 2012). It has been considered that immobilization with proper mineral nanomaterials is a good strategy to diminish the possible release of these nanoparticles into the environment, facilitating their manipulation (Tokarčíková et al., 2014). Concerning TiO_2 NPs, these have already been developed in conjugation with modified clays minerals, but to our knowledge there are no earlier toxicological studies on these novel nanocomposites (Manova et al., 2010).

In this context, the main goal of the present work was to evaluate in vitro cytotoxicity and genotoxicity of a nanocomposite (herein designated as C-TiO_2) constituted by rutile TiO_2 NPs and high purity nanokaolin (NK) clay in a hepatocellular carcinoma human cell line (HepG2). For a matter of comparison, both TiO_2 and NK single particles were also studied.

2. Materials and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red dye, resazurin, Triton X-100, 2-mercaptoethanol, low melting point (LMP) agarose, Tris hydrochloride (Tris-HCl) and methyl methanesulfonate (MMS) were purchased from Sigma-Aldrich Co. Dimethyl sulfoxide (DMSO), glacial acetic acid (CH_3COOH), absolute ethanol (C_2H_6O), sodium hydroxide (NaOH), sodium chloride (NaCl) and potassium hydroxide (KOH) were bought from Merck KGaA. Cell culture media components and SyberGold® were all Invitrogen™ and purchased from Thermo Fisher Scientific Inc. Lactate dehydrogenase (LDH) protein from rabbit muscle and Tris base were purchased from Calbiochem, while potassium chloride (KCl) and disodium salt dihydrate (Na_2EDTA) were purchased from Prolab. Finally, high purity 2-(4-Iodphenyl)-3-(4-nitrophenyl)-5-phenyltetrazoliumchlorid (INT) and normal melting point (NMP) agarose were supplied from VWR chemicals, Lonza Group AG and Bioline, respectively.

2.2. Nanomaterial preparation

The nanomaterials under study were rutile nanoparticles (NM-104, UE Joint Research Centre, Institute for Health and Consumer Protection), high purity kaolin nanoclay (Nanobiomatter Industries S.L.) and rutile nanoparticles immobilized in high purity kaolin nanoclay. The rutile TiO_2 NPs were dispersed and anchored on NK clay to form nanocomposite, C-TiO_2, following the method patented by Fernandez et al. (2010), presenting 10% of rutile TiO_2 NPs and 90% of NK clay.

All studied nanomaterials were suspended in serum-free and complete HepG2 cell culture media (300 μg/mL for TiO_2 NPs, 2700 μg/mL for NK clay and 3000 μg/mL for C-TiO_2 nanocomposite). Afterwards, the suspensions were homogenized in a microfluidizer (Model LM10, Microfluidics International Corporation) at 12500 Psi, for three times. This procedure was performed in an interaction chamber with Y geometry (single-slotted) with suspensions placed in an ice-water bath.

Prior to each toxicity treatment and interference analysis, nanomaterials suspensions were sonicated in water bath for 5 min. Serial dilutions were carried out to obtain the different nanomaterial concentrations tested and sonicated in water bath for 5 additional minutes prior to testing.

2.3. Nanomaterial characterization

Primary particle size, size distribution and particle morphology were characterized by Field-Emission Scanning Electron Microscopy (FE-SEM) with a 1.5 nm resolution, working at 20 kV with a current intensity of 10 μA (Model S-4700, Hitachi Ltd. Corporation). Information on size distribution was calculated from measuring at least 100 nanoparticles in random fields of view.

Average hydrodynamic size, zeta potential and polydisperse index of suspensions were measured by DLS using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN 3600, Malvern Instruments Ltd.). For this evaluation, the nanomaterials suspensions were diluted to a 0.01 μg/mL concentration.

2.4. Cell culture

Hepatocellular carcinoma human cell line (HepG2) was obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in nutrient mixture DMEM (Dulbecco's modified Eagle's medium) medium with L-Glutamine (2 mM), antibiotic and antimycotic solution (100 units/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin), supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. To carry out the experiments, 3×10^4 cells were seeded in 96-well plates and 2×10^5 cells in 24-well plates for the cytotoxic and genotoxic evaluation, respectively, and allowed to adhere for 24 h at 37 °C. For cell treatments, these were incubated at 37 °C for different periods in the presence of the different nanomaterial concentrations, or control solutions.

2.5. Exposure conditions

For each cytotoxic assay, nanomaterial and type of cell culture media (complete and serum-free), 6 different concentrations of each nanomaterial (5, 25, 50, 100, 200 and 300 μg/mL for TiO_2 NPs; 45, 225, 445, 900, 1800 and 2700 μg/mL for NK clay and 50, 250, 500, 1000, 2000 and 3000 μg/mL for C-TiO_2 nanocomposite), in addition to a negative and a positive control, and 3 exposure periods (3, 6, and 24 h) were evaluated. Complete or serum-free media was used as negative control

and Triton X-100 (1%) in complete/serum-free media were used as positive control.

For genotoxic assessment, 3 non-cytotoxic concentrations of each nanomaterial were tested (5, 25 and 50 μg/mL for TiO_2 NPs; 45, 225 and 445 μg/mL for NK clay and 50, 250 and 500 μg/mL for C-TiO_2 nanocomposite) in both complete and serum-free media, for 2 periods of exposure (3 and 24 h). Complete/serum-free media were used as negative control and MMS 100 μM in complete/serum-free medium was used as positive control. After treatment, the cells were washed with PBS and detached with trypsin 0.25% for 1 min, which was then inactivated with complete medium. The content of each well was collected into a microtube and centrifuged for 3 min at 7500 $\times g$ (for three times). After the first centrifugation the pellet was suspended in PBS. After the second centrifugation, the cell density of 2.5×10^4 cell/mL was calculated and transferred to another microtube for the final centrifugation.

2.6. Interference studies of nanomaterials with cytotoxic assays

To reveal possible interferences of nanomaterials with cytotoxicity assays experimental procedures, two main sets of experiments were conducted: (1) to analyse the effect of materials presence on optical measurements (light-absorption interference) and (2) to assess possible nanomaterials reactivity with assay components (catalytic interference). For the LDH assay, an additional experiment was carried out to understand the possible interference of nanomaterials on the enzymatic activity of LDH. All sets of experiments were carried out in the absence of cells; the three highest concentrations of each material were tested (25, 100 and 300 μg/mL for TiO_2 NPs; 0, 225, 900 and 2700 μg/mL for NK clay and 250, 1000 and 3000 μg/mL for C-TiO_2 nanocomposite) and complete/serum-free media was used as negative control.

2.6.1. Light-absorption interference.

For MTT reduction and neutral red uptake (NRU) assays, light absorption interference was analysed in the presence of dissolving solutions (DMSO and fixative solution, for MTT and NRU assay, respectively) and reaction end-products (formazan dissolved in DMSO and neutral red dissolved in fixative solution) as previously suggested by Kroll et al. (2012). End-products were obtained by incubating live cells with MTT and NRU dyes. The nanomaterials suspensions were prepared in dissolving solutions and end-products at the concentrations mentioned above. In turn, for the alamar blue (AB) assay, the nanomaterials suspensions were prepared in both resazurin and its pink-coloured reduced form resorufin obtained by reaction with formic acid.

The interference of nanomaterials presence in optical detection of INT reduction during the LDH assay was performed using oxidized and reduced INT. The reduced INT was obtained by adding 2-mercaptoethanol until obtain a red colour. Total reduction of INT was verified as previously described by Kroll et al. (2012).

Absorbance was measured at 570 nm and 540 nm for MTT and NRU, respectively, at 570 nm with a reference wavelength of 630 nm for the AB assay, and at 490 nm with a reference wavelength of 655 nm for the LDH assay using a Cambrex ELx808 microplate reader (Biotek, KC4).

2.6.2. Catalytic interference.

Nanomaterials reactivity with dyes was determined by incubating 100 μL of nanomaterials suspensions prepared in complete and serum-free media with MTT, NRU and AB assays reagents in the absence of cells (4 h of incubation for MTT and AB, and 3 h for NRU). The absorbance was measured at the end of the incubation.

In case of LDH assay, positive control wells with only 100 mU of LDH were also included. The assay was carried according to the kit manufacturer's instructions (Cytotoxicity Detection Kit LDH, Cat. no. 11644793001, Roche Diagnostics Corp). As previously described by Kroll et al. (2012), light absorption was measured continuously at 490 nm with a reference wavelength of 655 nm using a Cambrex ELx808 microplate reader (Biotek, KC4) for 1 h at 37 °C.

2.6.3. Enzymatic interference (LDH activity).

For the LDH assay, an additional experiment was carried out to understand the possible interference of nanomaterials on the enzymatic activity of LDH, by either inactivating or adsorbing the LDH protein based on Ahmad et al. (2012), Kroll et al. (2012) and Holder et al. (2012). For each nanomaterial concentration different amounts of LDH (20, 50, 100 and 500 mU) were tested in a 96-well plate and four periods of exposure were assessed (15 min, 60 min, 3 and 24 h). After these time periods, 100 μL of the kit reaction buffer was added and the remaining assay was carried according to the kit manufacturer's instructions (Cytotoxicity Detection Kit LDH, Cat. no. 11644793001, Roche Diagnostics Corp). Spectrophotometric absorption was taken at 490 nm with a reference wavelength of 655 nm using a Cambrex ELx808 microplate reader (Biotek, KC4).

2.7. Cytotoxicity assays

At the end of the exposure period, the treatment was removed and 100 μL of MTT or AB reagent, prepared in serum-free medium (0.5 mg/mL and 20 μg/mL, respectively), were added to each well and incubated for 4 h at 37 °C in the dark. Regarding the MTT assay, after removing the MTT, the produced formazan was solubilized with 200 μL DMSO. Thereafter, the plates were centrifuged (Universal 320, Hettich) at 4000 $\times g$ for 10 min and 100 μL of the supernatant were transferred to a new plate for the final reading. Spectrophotometric absorbance was measured at 570 nm for MTT and at 570 nm with a reference wavelength of 630 for AB, using a Cambrex ELx808 microplate reader (Biotek, KC4).

2.8. Genotoxicity assessment

2.8.1. Interference study.

To evaluate the possible interactions of nanomaterials with alkaline comet assay, a lysis test was performed. This involved the addition of 100 μL of nanomaterials at the highest tested concentrations to an equal volume of cells suspended in 1.2% LMP agarose just before running the assay. The assay was then performed as described below in Section 2.8.2. Results obtained for each type of media (complete or serum-free) and each type of nanomaterial (TiO_2 NPs, NK or C-TiO_2) were compared with a negative control (cells without nanomaterials).

2.8.2. Alkaline comet assay.

Cells were embedded in 200 μL of 0.6% LMP agarose and 5 μL drops were placed on microscope slides precoated with 1% NMP (the technique was carried out using a medium throughput system 12 gel comet assay unit, Severn Biotech Ltd.®). After agarose solidification, the slides were placed in a coplin jar and immersed in lysis solution (NaCl 2.5 M, Na_2EDTA 100 mM, Tris-base 10 mM, NaOH 10 M, pH 10, Triton-X 100 1%) during 1 h. For unwinding of DNA, all slides were immersed in electrophoresis solution (Na_2EDTA 1 mM, and NaOH 0.3 M, pH 13) in the electrophoresis platform for 40 min, followed by electrophoresis for 20 min (1.15 V/cm). Slides were then transferred to cold PBS (pH 7.2) for 10 min and cold deionized water for 10 min. Slides were fixed with ethanol 70% and 96% for 15 min each at room temperature and gels were dried overnight. Slides were stained with SyberGold® in TE buffer (Tris-HCl 10 mM and EDTA 1 mM) and visualized in a Nikon Eclipse E400 microscope with an epi-fluorescence attachment and 250× magnification. Slides were analysed using the Comet Assay IV Software (Perceptive Instruments). At least 100 cells were scored (50 for each replicate gel). The percentage of DNA in the comet tail (% tDNA) was used as DNA damage parameter.

2.9. Statistical analysis

Statistical analysis was performed using SPSS for Windows statistical package (version 23.0). The continuous data from cell viability and the comet assay were tested for normality using the Kolmogorov-Smirnov test. Since it was not normally distributed, different mathematically transformations were attempted but normal distribution and variance homogeneity (tested with Levene's test) was only possible for comet assay data.

Therefore, cytotoxicity data was analysed using the non-parametric Jonckheere-terpstra test to examine possible trends along the tested concentrations. Comet assay data was analysed after data transformation (using ln function) using one-way ANOVA with Dunnet's test for pairwise comparisons and linear regression for trend analysis. Whenever a statistical significant trend was found, a curve fir analysis was also performed using polynomial regression.

For all experimental conditions tested, a minimum of three independent experiments were performed (in viability assays, each performed in triplicate). Experimental data were expressed as mean ± standard error and a P-value < 0.05 was considered significant.

3. Results

3.1. Nanomaterial characterization

Rutile TiO_2 NPs were found to be nearly spherical agglomerates with different particle size. The primary particle of TiO_2 NPs was mainly elongated and rounded, suggesting an ellipsoidal, rod-like 3D structure with <50 nm long. TiO_2 NPs were highly agglomerated. Regarding NK clay, its FE-SEM analysis showed irregular laminar particles having 0.1 to 1.4 μm in length and <100 nm in thickness. NK particles were agglomerated in spherical particles due to the spray drying procedure followed after purification of clay. Despite remaining spherical agglomerates of the nanoclay, the C-TiO_2 nanocomposites exhibited irregular laminar type shape, characteristic of the mayor clay component (Fig. S1).

Table 1 summarizes the main physico-chemical properties of the nanomaterials suspended in water, serum-free and complete media, namely hydrodynamic size, dispersity and zeta-potential. In all aqueous suspensions, TiO_2 NPs exhibited the smallest hydrodynamic size, when compared to the remaining materials while C-TiO_2 nanocomposite presented higher size than TiO_2 NPs and NK clay (with exception in the serum-free medium). In addition, C-TiO_2 tended to agglomerate more extensively in water than in either biological media, presenting the size of 7289 nm and a polydisperse index of 1; this means that the C-TiO_2 suspension is non-uniform in water. When in cell culture media, C-TiO_2 presented larger size in serum-free medium (2419 nm) than in complete medium (470 nm); however, dispersity was very similar for both media. Similarly to the nanocomposite, both TiO_2 NPs and NK presented higher hydrodynamic sizes in serum-free than in complete media.

Regarding measurements of zeta potential in water, the TiO_2 NPs showed the largest absolute value (ζ – potential of 41.6) and, therefore, was found to be the most stable suspension, while the C-TiO_2 nanocomposite was the least stable (ζ – potential of 10.4).

3.2. Nanomaterial interferences with in vitro cytotoxicity assays

Table 2 represents the main interferences observed in the present study. Light-absorption interferences studies intended to analyse the influence of materials presence on absorbance measurements while catalytic interferences addresses possible nanomaterials reactivity with assay components. For the LDH assay, an additional experiment was conducted, evaluating the interference of nanomaterials on LDH enzymatic activity.

The results obtained showed that all tested materials, regardless of the presence or absence of serum, did interfere with absorbance measurements, both in presence of the substrate and final products of each assay introducing bias in the final result; one exception was observed for TiO_2 NPs in the LDH membrane integrity assay. To obviate this interference due to nanomaterial presence in assay reading, the plates were centrifuged before absorbance measurements. This procedure was enough to eliminate optical interference of these nanomaterials for MTT, AB and NRU assays since, after centrifugation, the absorbance obtained was similar to those obtained in negative controls (data not shown). However, for LDH assay, the centrifugation was not enough to eliminate the NK and C-TiO_2 light interference, as a decrease in absorbance was still observed with the increase in nanomaterial concentrations (data not shown).

Regarding the nanomaterials reactivity with the assay components, all nanomaterials concentrations exhibited a similar signal compared to negative controls for the MTT, AB and LDH assays in both media, which ruled out catalytic interferences from nanomaterials under evaluation. In opposition, during NRU assay, the NK clay and C-TiO_2 nanocomposite could react with the NRU assays components, possibly by adsorption, leading to a significant decrease in absorbance signal.

Since LDH is an enzymatic assay, it was also evaluated if studied nanomaterials could inactivate or adsorb LDH protein present in the supernatant, which would decrease the final measured absorbance. In the absence of serum proteins, all nanomaterials were found to interfere with enzymatic activity of LDH over time, decreasing the final absorbance values.

The results obtained herein suggest that NK clay and C-TiO_2 nanocomposites were able to significantly interfere with NRU and LDH assay components, decreasing the signal in both assays. As these interferences could not be eliminated by protocol alterations, only MTT and AB assays were found to be suitable for further in vitro cytotoxicity studies after inclusion of a centrifugation step. In this way, we were able to assure that in the presence of cells there would be no

Table 1
Physicochemical description of studied nanomaterials (TiO_2 NPs, NK clay and C-TiO_2).

		DLS		LDV	
		Average hydrodynamic size (nm)	Polydisperse index	ζ – potential	pH
TiO_2 NPs	H_2O[a]	196.7	0.308	41.6	6.63
	HepG2 serum-free medium[a]	447.2	0.635	[b]	7.58
	HepG2 complete medium[a]	236.6	0.223	[b]	7.80
NK	H_2O[a]	508.4	0.244	−39.0	6.86
	HepG2 serum-free medium[a]	2675.0	0.617	[b]	7.72
	HepG2 complete medium[a]	447.5	0.474	[b]	7.91
C-TiO_2	H_2O[a]	7289.0	1.000	10.4	6.56
	HepG2 serum-free medium[a]	2419.0	0.433	[b]	7.62
	HepG2 complete medium[a]	470.0	0.471	[b]	7.82

DLS: dynamic light scattering; LDV: laser Doppler velocimetry.
[a] All materials were tested at 0.01 μg/mL.
[b] Media components did not allowed a correct assessment of the ζ – potential of materials.

Table 2
Interference of the nanomaterial, suspended in serum-free (SFM) and complete (CM) media, with in vitro toxicity test systems (MTT, AB, NRU and LDH assays).

		TiO_2 NPs SFM/CM	NK clay SFM/CM	C-TiO_2 SFM/CM
MTT	Optical	+/+	+/+	+/+
	Catalytic	−/−	−/−	−/−
AB	Optical	+/+	+/+	+/+
	Catalytic	−/−	−/−	−/−
NRU	Optical	+/+	+/+	+/+
	Catalytic	−/−	+/+	+/+
LDH	Optical	−/−	+/+	+/+
	Catalytic	−/−	−/−	−/−
	Enzymatic	+/−	+/−	+/−

+: interference detected.
−: no interference detected.

significant interferences of light or assay components with nanoparticles and therefore results obtained would not be biased by the nanoparticle presence but would be due to nanoparticle effect on cell function.

3.3. In vitro cytotoxicity

Regarding the cellular viability analysis using the MTT assay (Fig. 1), in serum-free medium, TiO_2 NPs induced a significant concentration-dependent decrease in viability of the liver cells only after 24 h of exposure to these nanoparticles ($P = 0.011$) in serum-free medium, while NK clay and C-TiO_2 nanocomposite induced a significant concentration-dependent decrease in viability of HepG2 cells, in all studied exposure periods. In complete medium, a significant concentration-dependent decrease in viability of HepG2 cells was observed in all studied periods of exposure and all materials with exception to 6 h of exposure to NK although in this case, P value was near to significance value (data not shown) (Fig. 1).

The results of cellular viability assessed by the AB assay (Fig. 2), were found to be similar to the ones obtained in the MTT assay in serum-free medium: TiO_2 NPs induced a significant concentration-dependent decrease in HepG2 viability only after 24 h ($P < 0.001$), while NK clay and C-TiO_2 nanocomposite lowered the cell viability for all periods of exposure in a concentration-dependent way. In complete medium, TiO_2 NPs exhibited a decrease in the HepG2 cell viability for the 3 and 6 h of exposure; for the 24 h period, it was not observed a statistically significant trend, possibly due to the increase in cell viability observed at 100 μg/mL concentration point.

Once again NK and C-TiO_2 nanomaterials presented similar results to MTT results (despite for 3 h the C-TiO_2 did not present a significance value).

3.4. Nanomaterial interferences with alkaline comet assay

Literature describes a mechanism involved in nanomaterials interference with alkaline comet assay, which includes association to nucleoid DNA, affecting its behaviour during electrophoresis and inducing breakings in the naked DNA. Therefore, during the lysis step of the comet assay, nanomaterials may interact with the unprotected DNA and cause additional DNA damage, interfering with the sensitivity of the assay, thus misleading the results (Karlsson, 2010; Magdolenova et al., 2012).

In serum-free media all nanomaterials seemed to cause additional DNA damage, interfering with the assay. Conversely, there were no significant differences between the lysis test of all materials and the negative control in the presence of serum proteins, showing that tested nanomaterials did not interfere with the alkaline comet assay (data not shown). To eliminate interferences observed in serum-free medium, extensive washing of the wells was performed after the nanomaterials exposure (just before performing the alkaline comet assay), in order to remove nanomaterials.

3.5. In vitro genotoxicity

In the absence of serum proteins it was observed a significant increase in DNA damage only after 3 h of exposure to TiO_2 NPs

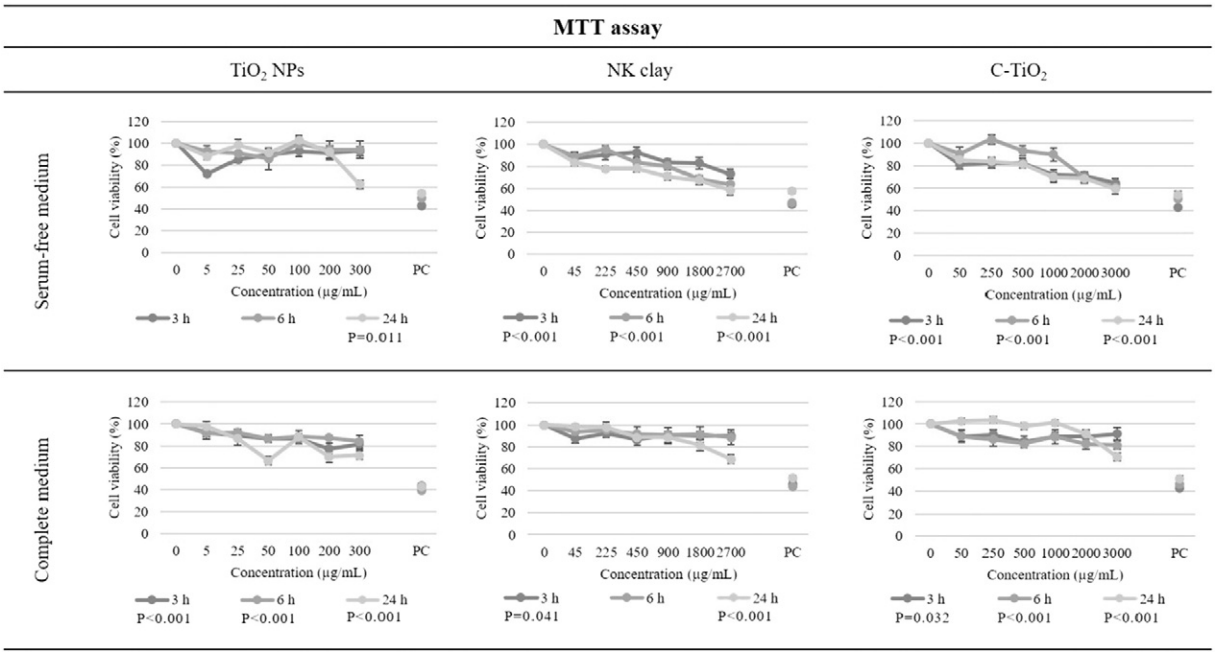

Fig. 1. Cell viability of the HepG2 cell line after exposure to TiO_2 NPs, NK clay and C-TiO_2 nanomaterials assessed by MTT viability assay in serum-free and complete media. Values were normalized considering negative control as 100%. P value < 0.05 significance difference after trend analysis using the Jonckheere-Terpstra test. PC: positive control (Triton X-100 1%).

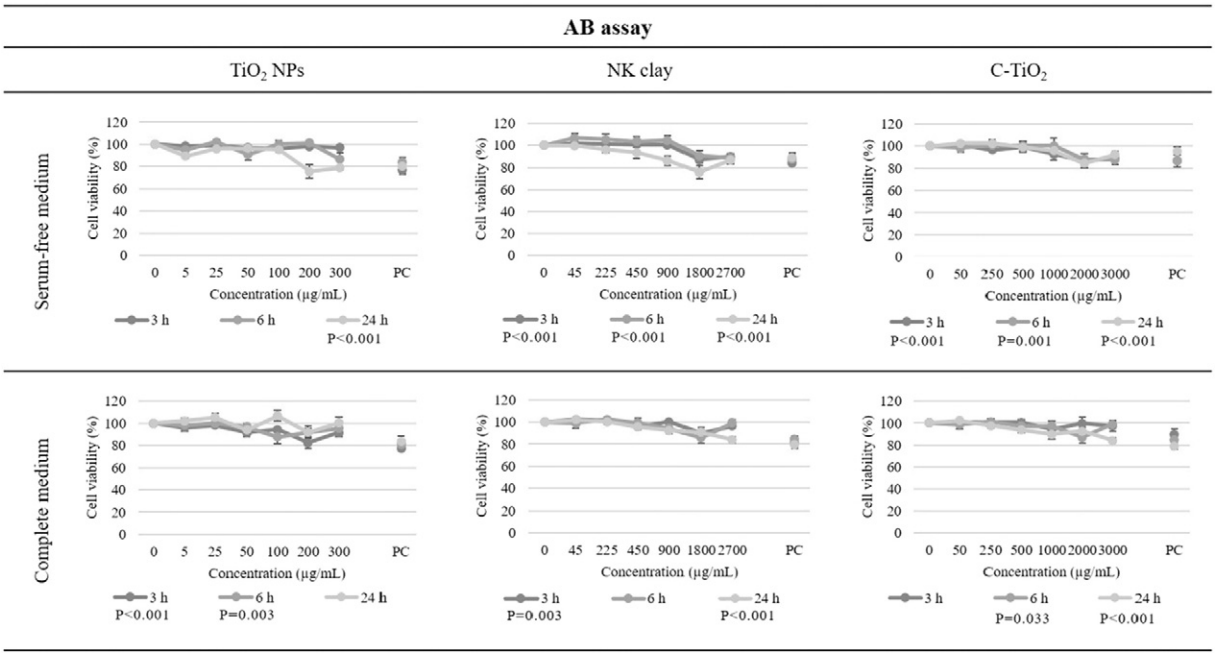

Fig. 2. Cell viability of the HepG2 cell line after exposure to TiO_2 NPs, NK clay and C-TiO_2 nanomaterials assessed by AB viability assay in serum-free and complete media. Values were normalized considering negative control as 100%. P value < 0.05 significance difference after trend analysis using the Jonckheere-Terpstra test. PC: positive control (Triton X-100 1%).

(Fig. 3). On the other hand, the opposite phenomenon was observed in the presence of serum proteins, i.e., it was observed DNA damage only after longer periods of exposure (24 h).

Concerning NK and C-TiO_2 nanomaterials, it was observed that both materials presented a similar impact on DNA integrity in each of the studied cell culture media. These nanomaterials could induce significant single strand breaks and alkali-labile sites on the HepG2 DNA only after 3 h of exposure in complete medium (Fig. 3).

4. Discussion

One of the main concerns of nanotechnology applications is that nanoparticles may be more toxic, and therefore more hazardous than larger particles and bulk materials, due to their large surface area and consequent enhanced chemical reactivity and also cellular internalization (Tucci et al., 2013). In the past years, TiO_2 NPs have been extensively studied to investigate their potential toxicological effects due to their

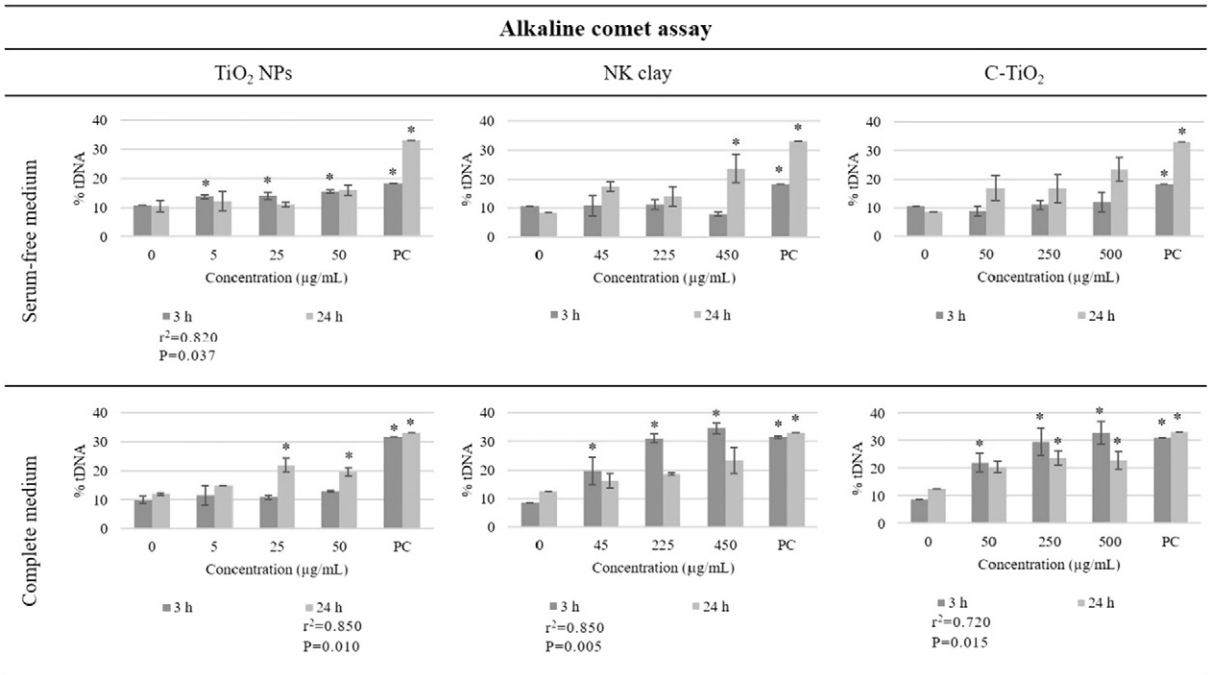

Fig. 3. Genotoxicity of TiO_2 NPs, NK clay and C-TiO_2 nanomaterials on HepG2 cells analysed by alkaline comet assay in serum-free and complete media. P < 0.05 significant value after analysis with single pair-wise Dunnet's test and R^2 coefficient of determination after curve fit with polynomial regression. *P value < 0.05. PC: positive control (MMS 100 μM).

increased environmental and occupational exposure (Tucci et al., 2013). In order to monitor and control the release of TiO_2 NPs, the technique of immobilization of these nanoparticles into crystalline substrates, such as inorganic clay minerals, was developed to facilitate their manipulation, restraining their release and diminishing their threat into the environment and human health (MODENA, 2012; Tokarsky et al., 2012; Tokarčíková et al., 2014).

The present study measured the cellular interaction and biological responses of human hepatocytes after exposure to TiO_2 NPs immobilized in NK clay laminar structures after characterization of these nanomaterials.

Our results suggest that the absence of serum leads to an increase in the agglomeration state, i.e., serum proteins allow a better dispersion of these materials. When attempting to perform zeta potential measurements, it was found that the ionic strength of medium impacted measurement reproducibility since the high salt content would interfere with the electrodes in the capillary tube. Additionally, in the presence of FBS, a very broad distribution of charges with a peak close to 0 mV was found due to the wide distribution of charges from both particles and proteins as observed in previous studies (Murdock et al., 2008).

To achieve the main goal of the present work, a set of experiments was performed in order to assess cellular viability and DNA damage on hepatic cells. In addition, all proposed assays were tested in regards to possible interference of studied nanomaterials with optical, catalytic and even enzymatic performance. Only the MTT and AB assays were found to be suitable for cytotoxicity assessment; while MTT allows the detection of mitochondrial activity (Kroll et al., 2009), AB is an indicator of the redox potential of cells (Takhar and Mahant, 2011). Both NK clay and C-TiO_2 nanocomposite exhibited light and catalytic interferences for NRU assay regardless the presence of serum, while all materials interfered with LDH enzymatic activity when suspended in serum-free medium. Possibly, LDH proteins were adsorbed to materials surface and ceased to be able to reduce the INT compound. This would trigger false negatives, i.e., the assay would indicate that the integrity of the cellular membrane was maintained when, in reality, the LDH was simply not available to convert lactate to pyruvate due to nanomaterials adsorption. Such an effect was not observed in complete medium (with FBS proteins), possibly due to the presence of a protein corona formed by the FBS proteins around the materials, which avoided LDH adsorption. These results were in accordance with some previous interferences studies in different metal nanoparticles, with special emphasis on TiO_2 NPs, which also demonstrated that LDH could be adsorbed and even get inactivated by these nanomaterials (Ahmad et al., 2012; Guadagnini et al., 2013).

Actually, there are reports that already indicate that metal oxide nanoparticles interact with assay components or dyes or even with optical readouts due to high adsorption or scattering by nanoparticles, distorting the assay outcome on the viability assessment (Kroll et al., 2012). A recent work reported that TiO_2 NPs could interfere with the NRU assay, increasing the signal reading for these dyes, which could be related to the light-absorbing properties of this nanoparticle (Guadagnini et al., 2013). Notwithstanding this, the signal in the presence of NK and C-TiO_2 was found to be lower in the NRU assay for our current study, which could indicate that these nanomaterials could reacted with the NRU assay components, possibly adsorbing to them. This would decrease the signal with increase of material concentration, misleading the results. In respect of NK or even C-TiO_2, no interference studies regarding the toxicity assays have been made so far.

Iavicoli et al. (2011) reviewed studies on the cytotoxicity of TiO_2 NPs, but only few reported analyses of rutile TiO_2 NPs effects on hepatic cell lines. According to literature, TiO_2 NPs rutile:anatase can induce in vitro cytotoxicity on hepatic cells after long periods and high doses of exposure. For instance, Gaiser et al. (2013) found no cytotoxic on C3A hepatocytes after exposure to rutile with minor anatase TiO_2 NPs up to high doses (approximately 2000 μg/mL), using the AB and LDH assays. These results were consistent with the ones obtained by Kermanizadeh et al. (2013) for the same cell line and using exclusively mitochondrial activity assays. With respect to studies performed on HepG2 cell line, Prasad et al. (2014) demonstrated that TiO_2 NPs (mainly constituted with anatase) did not induce a significant decrease in cell viability in the presence of FBS, at concentrations below 100 μg/mL even after 24 h of exposure. Furthermore, Shukla et al. (2013) found only a negligible loss of HepG2 viability after 6 h of exposure while, for longer time periods (24 and 48 h), concentrations of 20 to 80 μg/mL induced a significant decrease on viability of approximately 20%, assessed with MTT and NRU assays. In opposition to those results, our rutile TiO_2 NPs exhibited adverse effects on the hepatocytes mitochondrial activity for short exposure periods in complete medium, shown by the decreased viability observed in the MTT assay.

In the present work, rutile TiO_2 NPs induced an increase on DNA damage of HepG2 cells only for the shortest periods of exposure (3 h) in incomplete medium; the lack of effect observed for longer periods of exposure (24 h) may be due to DNA repair or diminished nanoparticle internalization associated to agglomeration phenomena (nanomaterials internalizations results are not shown). In complete medium, higher levels of DNA damage were observed after 24 h of exposure, suggesting that for shorter periods of exposure, the presence of serum proteins may protect DNA from TiO_2 NPs effect. According to the Nanogenotox project report, which evaluated the genotoxicity of different TiO_2 NPs from the JRC repository in different cell lines (BEAS 2B, 16 HBE, A549, Caco-2 and NHEK), the NM-104 TiO_2 NPs studied here only caused DNA damage on the dermal cell line NHEK, again after 3 and 24 h of exposure; however, for the same concentrations tested in the present work, in the NHEK cell line there were not found any significant DNA damage (Norppa et al., 2013). Different projects studying rutile and anatase TiO_2 NPs concluded that anatase TiO_2 NPs induced more extensive DNA damage than the rutile ones in HepG2 cell line (Petković et al., 2011). This has led to some authors to suggest that rutile TiO_2 NPs could be considered as negative controls in nanotoxicological studies (Liu et al., 2013), which is not supported by our results.

Despite the multiple functionalities of the clay minerals, the use of nanoclays still raises concerns due to the little information available about the toxicity that these nanomaterials can induce (Maisanaba et al., 2015). Herein, the NK mineral induced a significant concentration-dependent decrease in the viability of HepG2 cells for all periods of exposure and media types (with exception of the 6 h treatment in complete medium for both assays). According to our knowledge, no previous studies on HepG2 cell have previously been made with this type of clay and there are still few toxicological studies in the literature about clays with this hepatic cell line (Abdel-Wahhab et al., 2015). Different cytotoxicity studies using clays demonstrated that these minerals are able to cause cytotoxicity in different cell lines, and even induce cell death (Maisanaba et al., 2015). Some studies show that several clays induce cytotoxicity only at high concentrations (Han et al., 2011) while others describe significant loss of cellular viability at lower concentrations (Lordan et al., 2011; Verma et al., 2012; Maisanaba et al., 2014).

Regarding the C-TiO_2 nanocomposite, the results observed in the comet assay were found to be similar to the ones obtained for NK clay: higher levels of DNA damage were observed only after shorter periods of exposure (3 h) in complete medium possibly due to diminished nanoparticle internalization at 24 h (nanomaterials internalizations results are not shown). The exact same effect was observed in both materials, probably because C-TiO_2 nanocomposite is mainly constituted by NK clay. When comparing results obtained in complete and incomplete media, it seems that the presence of serum proteins promotes the internalization of both NK clay and C-TiO_2 nanocomposite (data not shown), which can possibly be related to the capacity of these materials to interact with HepG2 DNA, causing further damage. The interaction of nanomaterials with serum proteins is often related with the formation of a protein corona that, in some cases, is associated with cell uptake. Once inside the cells, nanomaterials may

disrupt the mitochondrial function, as well as, inducing the production of reactive oxygen species, possibly leading to DNA damage (Albanese et al., 2012). Other authors studying genotoxicity of different type of clays also observed DNA strand breaks on hepatic HepG2 cells in a time dependent manner, which is further confirmed by comet assay (Maisanaba et al., 2013; Houtman et al., 2014).

Altogether, results suggest that NK clay has the largest contribution to the cytotoxicity and genotoxicity of C-TiO_2 nanocomposites. Possible synergistic or additive effects between the studied materials are not discussed as our study design is not the most appropriate to understand the real interactions that may occur. Thereby, NK clay is not a suitable clay substrate for the immobilization of other nanomaterials (for instance the TiO_2 NPs), since increased concentrations of NK induce a significant decrease in the cell viability in the HepG2 cell line, which was also observed on the C-TiO_2 nanocomposites. A possible strategy to overcome the cytotoxic and genotoxic effects of naked kaolinite observed in this study is to chemically modify NK clay with organic compounds (Maisanaba et al., 2014). These data underline that further toxicity studies must be performed in organoclay particles to understand if the chemical modification allows the creation of a more compatible NK clay.

5. Conclusions

Both cytotoxic and genotoxic effects were observed on the HepG2 hepatic cell line after exposure to C-TiO_2 nanocomposites and its single elements. Results here obtained confirm that TiO_2 nanoparticles present toxicity to HepG2 cells and that the immobilization of these nanoparticles in NK clay substract is not enough to diminish its toxicity. In fact, we could observe that NK clay is also responsible for cytotoxic and genotoxic effects in the studied hepatic cell line being the major contributor for the toxicity found also for the nanocomposite. Taking all into account, the present study suggests that NK clay is not a suitable mineral substrate for the immobilization of other nanomaterials.

The current innovative study represents a primordial toxicity evaluation of the nanocomposites using mineral nanoclay structures to tame NPs toxicity on environmental and human exposure, moving towards to a safer application of these new materials.

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Conflict of interest statement

The authors declare that the original work described is approved by all co-authors, has not been previously published and is not under consideration for publication elsewhere. In addition, the authors also declare that they have no conflicts of interest concerning this article.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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